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STAINING AND PERMANENT PRESERVATION OF HISTOLOGICAL ELEMENTS ISOLATED BY MEANS OF CAUSTIC POTASH (KOH) OR NITRIC ACID (HNO_3).*

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As pointed out long ago by Moleschott (16), "properly chosen isolating reagents in the hand of the histologist form the best kind of a knife." Two of the most efficient of these isolating agents are Caustic Potash (*potassium hydrate*, KOH), of 30 to 50 per cent., and Nitric Acid (HNO_3) of 20 per cent. aqueous solutions. Their action is somewhat similar, dissolving or rendering soft and non-resistant intercellular or ground substance, and acting more quickly on connective tissue than on cell cement. Consequently they serve for the isolation of glandular elements, like the tubules of the kidney, gastric glands, acini of the salivary glands, etc., or by a somewhat more prolonged action for the isolation of the individual histological elements. If the action is unchecked, both agents finally destroy all the cellular elements also.

The real service of these reagents in histology is, therefore, the very important one of enabling the investigator to determine the form and relations of the structural elements, but when the more intimate structure is to be determined it can be more successfully accomplished, in most cases, by other methods.

Both substances have been used in animal and vegetable histology for a long time, but the principles underlying their successful and practical employment in animal histology were only pointed out in 1846 and 1848.

In 1846 Donders (4) demonstrated the remarkable fact that caustic potash in concentrated solutions dissolves connective tissue and cell cement with great readiness, but leaves the form of the cel-

*The bibliography is referred to by numbers corresponding to the titles.

lular elements intact for a considerable time, while weak solutions dissolve all the soft parts with great rapidity. Donders also pointed out the excellence of a concentrated potash solution for studying dried blood. Following this suggestion, Virchow (30), and later Woodward (33), made great use of a strong (35 to 40 per cent.) solution in the medico-legal examination of blood stains.

Twelve years after Donders' publication, *i. e.*, in 1858, Moleschott (16) experimented with various percentages of caustic potash, and finally decided that a 32.5 per cent. solution was the most generally useful, especially for muscular fiber cells (17). He also spoke favorably of a 35 per cent. solution, and as this is what is most commonly employed at the present day, the caustic potash method is often credited to Moleschott; but the fundamental and important discovery, viz.: that strong solutions isolate and preserve the structural elements for a considerable time, undoubtedly belongs to Donders.

In 1848 Paulsen (21) clearly demonstrated that a 20 per cent. solution of nitric, also of hydrochloric, acid gave the best results at the ordinary temperature for isolation, especially for muscular tissue. In 1849 Reichert (24) adopted Paulsen's method for the study of the musculature of the blood vessels, and thus gave it great popularity. Reichert, however, gave Paulsen the credit of having discovered the method. Many writers ascribe the discovery to Reichert, while still others credit it to Kölliker.

As stated above, while these two reagents first dissolve or greatly soften intercellular substance, and unless the action is checked, they finally destroy the cellular elements also, caustic potash acting much more rapidly than nitric acid. With nitric acid the action is mostly arrested by simply displacing the acid with water. The isolated elements may then be preserved indefinitely in glycerine, but not satisfactorily in alcohol. With caustic potash there was no known method of rendering the protoplasmic cellular elements permanent, as the action could not be checked. The addition of water, alcohol or glycerine simply dilute the solution, and the elements are quickly dissolved.

With vegetable tissues, the cellulose walls resist the action of the potash, and it may be washed away with water; but it is better to

use an acid even here (3, 11, 28). Horny animal cells also resist the action of caustic potash.

The contribution of the writers to the methods of using these reagents consists in pointing out clearly the methods of checking completely the action of both reagents at will, of making the isolated elements permanent *en masse* in alcohol or glycerine, and of successfully staining the elements and mounting them according to any of the approved ways employed by histologists. Part of this cantribution has already appeared in the proceedings of this Society or in the microscopical journals.* But it seemed worth while to bring together the best methods and the new discoveries with reference to these invaluable dissociating reagents, so that they might be more readily available for the use of students and investigators.

Caustic Potash (Potassium Hydrate, KOH).

1. Weak solutions destroy or dissolve all soft organic structures with great rapidity.

2. Strong solutions (30 to 50 per cent., also saturated solutions in water) act with great rapidity on intercellular substance and quite slowly on the cellular or structural elements, so that they may be isolated and studied in their natural forms and relations.

3. By the addition of water, glycerine or alcohol to the caustic potash upon the elements the solution is simply weakened, when it rapidly dissolves all the elements.

4. The action of the strong solution may be checked at any time (*a*) most satisfactorily by displacing it with a 60 per cent. solution of potassium acetate,† or (*b*) by the addition of sufficient glacial acetic acid to neutralize the caustic potash and form acetate of potash. After the action of the caustic potash is checked, the elements may be preserved indefinitely *en masse* in a 60 per cent. solu-

*The use of alum water for caustic potash preparations, and the subsequent staining in alum carmine, etc., is pubished here for the first time.

†In carrying on an investigation upon the cardiac muscles of the mammals for his graduation thesis, Mr. Boardman L. Oviatt, to whom the senior author had explained the action of glacial acetic acid in checking the action of the caustic potash, by a happy inspiration, used a strong solution of acetate of potash directly to displace the caustic potash. Subsequent mounting in glycerine was entirely successful, and the unavoidable shriveling due to the action of the strong acid was avoided. By later experiments, the senior author determined the proper percentage to employ, and has so far perfected the method that it is now as easy to employ as any of the methods in modern histology.

tion of acetate of potash, or, after being treated with a saturated solution of alum (see below), in 40 per cent. alcohol or glycerine.

5. A 30 to 50 per cent. aqueous solution—preferably a 35 to 40 per cent. solution (caustic potash in sticks, 35 or 40 grams, water 65 or 60 cc.)*—may be used for the isolation of the structural elements after hardening the tissues with alcohol, chromic acid or a chromium salt, picric acid, etc. It requires a longer time for the isolation of hardened than of fresh tissues, and the results are not so satisfactory. The elements of almost any tissue may be successfully isolated by means of strong solutions of caustic potash, but it has been most successfully employed in the study of the epidermal and muscular tissues, especially the elements of cardiac and smooth muscular tissue.

6. When fresh tissues are employed it is especially necessary that they be perfectly fresh, and that, if from organs like the heart, where a great deal of blood is present, the blood should be washed away with water before the application of the reagent, as the strong caustic potash preserves the blood corpuscles, and their presence is detrimental to the clearness of the outline of the elements.

7. Only small pieces of tissue should be used (if the tissue is massive the pieces should not exceed half a cubic centimeter), and about fifteen to twenty times as much potash solution should be used as tissue.

8. After ten to fifteen minutes the tissue should be tested with dissecting needles every five minutes, in order not to prolong the action unnecessarily.

9. As soon as the elements separate readily, the caustic potash is poured off and a plentiful supply of a 60 per cent. solution of acetate of potash added (potassium acetate 60 grams, water 40 cc). This displaces the caustic potash and checks its action (7, 8, 19). The efficiency and rapidity of the action of the acetate is increased by the addition of 1 per cent. of glacial acetic acid to it.

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*If the stopper is thoroughly vaselined it will prevent the rapid disintegration if it is cork, and the cementing if it is glass.

10. After the caustic potash has been removed the elements may be mounted for the microscope in 60 per cent. acetate of potash, in glycerine or glycerine jelly.*

11. Staining and mounting the isolated elements:—After the caustic potash has been displaced the acetate of potash is removed, and a plentiful supply of a saturated aqueous solution of alum is added and allowed to remain for a considerable time, preferably twenty four hours or more (9, 20). The elements then stain very satisfactorily with haematoxylin or alum carmine. Other stains may also be successfully used. After staining, the elements may be mounted by any of the approved methods — in glycerine, glycerine jelly (which is perhaps best), Farrant's solution, or Canada balsam. After the use of the alum water, the elements may be preserved *en masse* in 40 per cent. glycerine or 40 per cent. alcohol, stained and mounted whenever desired.†

If the preparation is left in acetate of potash for a day or more, the cells stain well with alum carmine or hæmatoxylin without using the alum water. It is necessary, however, to wash away the acetate quickly with water, or simply to absorb it, otherwise there will be a multitude of crystals in the preparation. The use of alum water for the cardiac muscles of the frog is not so successful as with those of mammals. Staining directly from the acetate is quite successful.

Nitric Acid (Acidum Nitricum, HNO_3).

1. Nitric acid in various degrees of concentration has been used for the isolation of the structural elements by many different histologists, but it is due to Paulsen (21) that a 20 per cent. solution (strong nitric acid 20 cc., water 80 cc.) came into general use; and while it serves fairly well for many of the tissues, its greatest applicability is to the isolation of the structural elements of muscular tissue, especially the ordinary striated or skeletal muscle and smooth or unstriated muscular tissue. For the cardiac muscular tissue, caustic potash is greatly superior. (See above.)

*If a staining agent is like alum carmine or picrocarmine (7, 8) is added to the glycerine in which the elements are mounted they gradually become stained. But the staining is more satisfactory if the elements are treated directly with the staining agent.

†If the caustic potash has not all been washed away from the elements there will be a copius precipitate formed when the alum water is added. The caustic potash is removed much more quickly (in five minutes in most cases) if 1 per cent. glacial acetic acid is added to the potassium acetate solution. The neutral solution for mounting or permanent preservation has been tested for nearly a year with success. Whether the acidulated acetate would serve equally well for permanent preservation remains to be investigated.

2. As just stated, a 20 per cent. solution of nitric acid has been found the best isolating agent for the fibers of striated muscle. Perfectly fresh tissues are preferable, and in fact necessary, for obtaining the best results, especially in separating the fibers their whole length. If the tissue is not fresh the fibers become fragile and cannot be isolated throughout their whole extent.

3. In order that the fibers may remain straight, the muscle should be suspended in the acid with its natural attachments if possible (9). When that is impracticable the muscle should be extended on a piece of cork and pinned in position with vaselined pins. The vaseline prevents the corrosion of the pins.

4. If the object is to isolate fibers throughout their whole length, the fat and connective tissue on the surface should be removed either before the immersion in the acid or as soon as the acid has sufficiently softened the connective tissue so that it may be removed without two much dragging upon the specimen. When the fibers can be separated easily, the action is sufficient. The time varies according to the temperature. At the ordinary temperature of a living room from one to three days is sufficient for almost any muscle. If the connective tissue is very dense, the time may be increased. By using heat the action may be completed in a few minutes, but it is not so satisfactory, as the surface layers are too much and the interior layers too little affected.

5. When the action is sufficient the muscle is transferred to water to remove the acid. It is well to change the water several times.

6. If it is desired to study the fibers with reference to their form, length and relations, the manipulation should be as little as possible. A fascicle is chosen which frays easily with a coarse sewing needle. It is transferred to a slide with a drop of water, or if a yellow color is desired, a drop of glycerine tinged with picric acid. The fibers are then carefully separated with coarse needles under a dissecting microscope. The excess of glycerine is removed with blotting paper, and a drop of warm glycerine jelly is allowed to spread slowly over the preparation. The fibers are arranged with

needles and the slide allowed to cool until the glycerine jelly has a glutinous consistency. It is then covered with a warm cover. The partly-cooled glycerine jelly prevents the fibers from becoming entangled when the cover is put on (9).

7. If the nuclei are to be especially studied, the muscle remains in water until all the acid is removed. Then it is stained in Koch's tubercle stain, diluted four or five times with water, for twelve hours or more. A fascicle is then removed to a slide with 20 per cent. alcohol, containing sufficient picric acid to make it a lemon yellow color. This is gradually replaced by 50 per cent., and then by 95 per cent. alcohol. In the last the fibers are separated as described above, and after draining away the alcohol, clove oil collodion is added and the fibers finally arranged. As soon as sufficient evaporation has taken place to fix the fibers, a cover glass is coated with Canada balsam and placed upon the specimen. If the result is successful the nuclei are stained a brilliant red and the body of the fiber yellow. The transverse striæ are always very sharp and clearly defined after picric acid staining (9).

8. If it is not convenient to study the specimen at once, or if it is a large one, like the œsophagus, and is to be studied for a considerable time, the action of the acid may be checked by transferring the specimen from water to a saturated aqueous solution of alum. In this the specimen may remain for several days, or even two weeks, in a cool place without marked deterioration. The fibers are treated as under 6, or the fibers may be stained quite successfully with hæmatoxylin, and may then be mounted in any way desired (20).

9 If one cares only for the general structure of a muscular fiber, not caring for the length and relations, as in ordinary laboratory work, the muscle should be prepared as above in 4 and 5, washed with water, when the fibers separate easily, then transferred to a saturated solution of alum and allowed to remain a day or more. Then the fibers may be successfully stained with aqueous hæmatoxylin, mounted in glycerine, glycerine jelly, or Canada balsam, etc. If it is desired to preserve for future use a large amount of

this dissociated material, it may be placed in 40 per cent. glycerine or 40 per cent. alcohol from the alum water, and stained and mounted at any time; or the fibers may be shaken in a bottle of alum water till they are separated, then stained with hæmatoxylin and preserved *en masse* in 40 per cent. glycerine. This is a very convenient method for a large laboratory. Then a small amount of the dissociated material can be given to the students as they are ready for it, and they can mount the fibers in glycerine jelly or in balsam.

10. For muscular fiber cells (smooth or unstriated muscle) the muscular coat of the stomach, or any other organ composed mostly of muscular fiber cells, may be placed in the 20 per cent. nitric acid till the intercellular connective tissue and cell cement are sufficiently softened to allow the cells to be shaken apart. The acid is then washed away with water. Alum solution is added, in which the cells are shaken apart. After twenty-four hours or more, the alum is poured off and the cells stained with hæmatoxylin or alum carmine. After washing away the staining fluid with water the cells may be mounted in glycerine or glycerine jelly.*

Bibliography.

In the following bibliography, the books mentioned usually give a more or less complete account of both the reagents discussed in this paper. In the scientific papers referred to are detailed the special applications of one or both reagents. Features of special importance are noted at the end of the titles.

1. Beale, L. S.—How to Work with the Microscope. 5th ed. London, 1880.

2. Busk and Huxley.—Manual of Human Histology. An edited English translation of Kölliker's Gewebelehre. Two vols. See Kölliker, 11.

3. Dippel, L.—Grundzüge der Allgemeinen Mikroskopie. Braunschweig, 1885. Dippel says, p. 320, that the swollen vegetable preparations which have been acted on by caustic potash should be neutralized by dilute hydrochloric or acetic acid. See also 28.

4. Donders, F. C. — Mikroskopische und mikrochemische Untersuchungen thierschen Gewebe. Holländische Beiträge zu den anatomischen und physiologischen Wissenschaften. Herausgegaben von Dr. J. van Deen, Dr. F. C.

*The senior author has found it a great convenience to put a large number of these muscle cells into a small vial of glycerine jelly, then when a mount is desired the jelly is warmed and a drop of the mixture placed on the slide. It is sure to contain a great many of the muscle cells. This method is also very convenient for isolated ciliated and other cells.

Donders, und Dr. Jacob Moleschott. Utrecht und Düsseldorf, 1846–1848. The use of caustic potash in varying strengths and the preserving action of the strong solutions is discussed by Dr. Donders in Heft 1, 1846, pp. 55, 56.

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6. Frey, H.—The Microscope and Microscopical Technology. Translated and edited by G. R. Cutter. pp. 624. New York, 1880.

7. Gage, S. H.—Microscopical Notes—The Microscope, December, 1886. pp. 265–268. In this paper is announced the discovery that caustic potash preparations of soft animal structures, cardiac muscle, etc., could be made permanent and stained by checking the action of the caustic potash with glacial acetic acid.

8. Same.—Article Muscular Tissue in the Reference Hand-book of the Medical Sciences. Edited by Albert H. Buck. New York, 1887. Vol. V, pp. 59–75. In this article are given the methods for nitric acid and caustic potash preparations of muscular tissue, including the permanent preservation.

9. Gage, Mrs. Susanna Phelps.—Form Endings and Relations of Striated Muscular Fibers in the Muscles of Minute Animals (Mouse, Shrew, Bat and English Sparrow)—The Microscope. Vol. VIII, 1888, pp. 225–237 and 257–272. Five plates. In this paper, pages 261–263, are first given the methods of staining striated muscular fibers with Koch's red tubercle stain, of arranging the isolated fibers on the slide in glycerine jelly or in clove-oil collodion before applying the cover glass to avoid the disarrangement of the fibers. In this paper is also first announced the senior author's method of using a saturated solution of alum water to check the action of nitric acid and render the fibers stainable in hæmatoxylin.

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11. Goodale, G. L.—Physiological Botany. I. Outlines of the histology of phaenogamous plants. II. Vegetable physiology. pp. 499+36. New York, 1885. On pages 6 and 7, under *potash*, Dr. Goodale says: "A moderately strong solution of potassa is the most useful clearing agent that we have. After a mass of tissue—for instance, an embryo—has been acted on by a solution of potassa until it has become translucent it is to be cautiously subjected to the action of an acid, preferably acetic or hydrochloric, and then washed. Sometimes, however, the potassa renders the tissues too nearly transparent, in which case they may be slightly clouded by a little alum water."

12. Kölliker, A.—Handbuch der Gewebelehre des Menschen. 1st ed. Leipzig, 1852. I Vol., pp. 637.

13. KÖLLIKER, A.—Handbuch der Gewebelehre des Menschen. 6th revised edition. Leipzig, 1889. 2 vols.

14. LEE, A. B.—The Microtomists Vade Mecum. A Hand-book of the Methods of Microscopic Anatomy. pp. 424, London, 1885.

15. M'KENDRICK, J. G.—A Text-book of Physiology, including Histology, by Philipp Stöhr. See 27. Vol. I. New York, 1888. Vol. II, 1889.

16. MOLESCHOTT, JACOB. — Zur Untersuchungen verhornten Theile des menschlichen Körpers. Moleschott's Untersuchungen zur Naturlehre des Menschen und der Thiere. IV Band. Jahrgang, 1858. pp. 97–103. In this paper is first given the percentage solution of the strong potash for preserving as well as isolating the structural elements, viz.: 30 to 35 per cent. aqueous solution. Moleschott refers to the discovery of Donders as to the preservative action of strong solution of potash. See above 4.

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19. OVIATT, B. L.—Cardiac Muscle Cells in Man and in Certain other Mammals. Thesis for the degree of B. S. presented to the Cornell University, June, 1887. Published in the proceedings of the American Society of Microscopists, 1887. pp. 283–298 In this paper is the announcement of the possibility of making permanent preparations isolated by means of caustic potash, by displacing the caustic potash with potassium acetate.

20. PEARSON, LEONARD.—The Muscular Coats of the Œsophagus of the Domesticated Animals. Thesis presented to Cornell University for the degree of B. Agr. June, 1888, and published in the proceedings of the American Society of Microscopists, 1888, pages 128–139; in The Microscope, 1888, pages 361–370, and in the Journal of Comparative Medicine, Vol. X, 1889, pages 59–72. The article is illustrated by fifteen figures in the Journal of Comparative Medicine. In this paper are given the methods of isolating the muscular fibers of the œsophagus and the announcement of the senior author's recommendation of a saturated solution of alum water for checking the action of the nitric acid and rendering the specimen capable of investigation for a week or two; a great desideratum for an organ like the œsophagus.

21. PAULSEN, DR.—Observationes microchemicæ circa nonnullas Animalium telas. Doparti, 1848. p. 16, et seq. In this dissertation are given directions for the use of 20 per cent. nitric and hydrochloric acid for the dissociation of muscular tissue, especially the unstriated.

22. RANVIER, L.—Traité Technique d' Histologie. pp. 1109. Paris, 1875–1888. See also the following:

23. RANVIER, L.—Leçons d' Anatomie Générale sur le Système Musculaire. Paris, 1880. Ranvier says in 22 that is impossible to make caustic potash preparations permanent; and in this book (23) that it is impossible to stain them.

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30. VIRCHOW, R.— Uber die forensische Unterunchungen von trockenen Blut-flecken. Virchow's Archiv. Bd. XII, 1857. pp. 334. Donder's method of using strong solutions of potassium hydrate for soaking out blood-stains is adopted. See also Woodward, 33.

31. WHITMAN, C. O.—Methods of research in Microscopical Anatomy and Embryology. pp. 255. Boston, 1885.

32. WOODHEAD, G. S.—Practical Pathology. pp. 484. Edinburgh, 1883. Under "Caustic Potash," page 47, the author says: "Caustic potash, 40 per cent. solution is useful for isolating muscle cells, as in case of *myoma uteri*. This is very rapidly accomplished, seldom taking longer than from twenty minutes to an hour. Tease out, stain with picrocarmine, and mount in glycerine. If time is available, and a permanent preparation of the muscle cells from such growths is required, use nitric acid, etc." No directions are here given for getting rid of the caustic potash, and a trial according to the directions resulted in the rapid solution of the muscle cells.

33. WOODWARD, J. J.—The Application of Photography to Micrometry, with Special Reference to the Micrometry of Blood in Criminal Cases. Reprint from the Philadelphia Medical Times, 1876. Uses strong solution of caustic potash for soaking out blood stains. See Virchow, 30.